J Ind Microbiol Biotechnol (2003) 30: 369–374
DOI 10.1007/s10295-003-0058-9

ORIGINAL PAPER

K. Lakrod · C. Chaisrisook · D. Z. Skinner

Expression of pigmentation genes following electroporation of albino *Monascus purpureus*

Received: 14 January 2003 / Accepted: 15 March 2003 / Published online: 15 May 2003

Abstract A UV-induced albino strain of *Monascus purpureus* was subjected to electroporation in the presence of genomic DNA from a wild-type red strain of the fungus. Eight colonies expressed color after several weeks of growth. The growth rates of all eight color variants were significantly greater than the recipient and donor strains under some culture conditions. Spectrophotometric analysis of the pigments extracted from the color variants revealed the pigments had absorbance spectra different from the DNA donor strain. These color variants may have resulted from transformation with wild-type DNA, mutation reversion, or activation of alternative pathway(s) – i.e., new mutations – that resulted in pigment production.

Keywords Transformation · Pigment · Polyketide · Anka · Ang-kak

Introduction

Monascus purpureus is a homothallic fungus found on red rice (Anka or Ang-kak). Historically, the fungus has been used as a natural food colorant and in traditional medicine in Asia for centuries [5, 7, 12]. The fungus is used in the production of fermented food products [11], and as a substitute for nitrite in meat preservation [8]. *M. purpureus* is also of interest from a medical standpoint because it produces monacolins, implicated in inhibition of cholesterol biosynthesis [15]. The color of the fungus varies from yellow to orange to red to purple-red, depending on culture conditions [5, 20]. The chemical structures of *Monascus* pigments are known. However, the biochemical pathways and genes controlling pigment production in *Monascus* are not known. Most research on *Monascus* has focused on growth substrates and on optimal conditions applied to different types of fermentation systems to enhance pigmentation [1, 16, 17, 20, 22, 23]. Other research has dealt with *Monascus* production of other metabolites, such as antibiotic compounds [19, 21].

The research reported here was directed toward developing *M. purpureus* strains that can be used to eventually identify genes involved in pigment production. In other efforts directed towards identifying genes of which there is little prior knowledge, workers have used transformation of a mutant recipient strain lacking a desired trait with total genomic DNA from a donor strain expressing the desired trait. For example, this approach was used with the Archaea *Halobacterium volcanii* where auxotrophs were transformed to prototrophy with wild-type genomic DNA [6]. Pulsed-field gel electrophoresis was used to examine restriction fragments of *Haemophilus influenzae* laboratory strains that had resulted from random uptake of large fragments of genomic DNA from donor strains [3].

Genomic DNA-mediated transformation has been successfully applied to fungal species. Genomic DNA uptake and expression has been demonstrated in *Trichoderma harzianum* and *Gliocladium virens*, where both biolistic and protoplast-mediated methods were used with fungal genomic DNA carrying a hygromycin resistance gene as the transforming DNA [14]. To identify fungal genes, an ''instant gene bank method'' in which a mutant recipient strain is transformed with genomic DNA from a wild-type strain, along with a helper plasmid carrying a fungal origin of replication, was proposed [2, 9]. A major advantage of this approach is that it bypasses the need for the construction of gene

K. Lakrod · C. Chaisrisook
Department of Microbiology, Faculty of Science,
Kasetsart University, Bangkok, Thailand

D. Z. Skinner (✉)
USDA-ARS and Department of Crop and Soil Science,
Washington State University, Pullman, WA 99164–6420, USA
E-mail: dzs@wsu.edu
Tel.: +1-509-3358696
Fax: +1-509-3352553

Present address: K. Lakrod
Mission College, Muak Lek, Saraburi, Thailand

libraries [2, 9], which is a time-consuming and difficult process [2]. However, the method relies on recombination between the helper plasmid and linear wild-type DNA during co-transformation, and the resulting transformants were frequently mitotically unstable [2, 9]. Kelly et al. [13], working with transformation of *Zalerion arboricola*, reported that including an origin of replication in the transforming plasmid increased the frequency of transformation, but also resulted in the occurrence of numerous unstable transformants. We attempted transformation of an albino mutant of *M. purpureus* with wild-type genomic DNA, but did not provide a plasmid carrying an origin of replication. This approach relies on origins of replication that exist in the recipient or introduced DNA. Similar studies have resulted in either no transformants [9] or only a few potential transformants [2]. However, those studies employed auxotrophic complementation and positive selection for the transforming DNA, requiring immediate expression of the introduced gene, with one exception [2]. In our case, only color expression was used, hence there was no selection pressure placed on introduced DNA. Consequently, any introduced DNA was allowed virtually unlimited time to stabilize and for transcription and translation to begin.

The specific objective of this study was to derive color-expressing isolates from the albino mutant to provide material for the identification of specific genes involved in pigmentation of this food fungus.

Materials and methods

Organism and cultivation

M. purpureus ATCC16365 (type-culture) was used as the source of wild-type genomic DNA. The UV-induced albino mutant KB20M1 [24] was used as the recipient in electroporation experiments. Cultures were maintained on modified complete medium containing 0.5% sucrose, 0.6% yeast extract, and 0.6% casein hydrolysate at 28°C in the dark. Mycelial masses for DNA extraction and protoplast preparation were harvested from liquid culture and incubated at 28°C on a rotary shaker at 180 rpm under 12-h illumination. The liquid cultures were blended in a sterile Waring blender at the highest speed for 30 s and transferred to fresh medium twice to increase the number of hyphal tips of the young mycelia. Mycelial masses were harvested by suction filtration through sterile Miracloth (Calbiochem, San Diego, Calif.) and washed three times with sterile distilled water before use in DNA extraction and protoplast preparation.

Monoconidial cultures derived from strains ATCC16365 and KB20M1 were grown on conidiation medium (100 g sucrose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g $NaNO_3$, 0.5 g KCl, 0.01 $FeSO_4 \cdot 7H_2O$, 3 g yeast extract, 5 g casamino acid and 1.5% agar per 1 l medium) at 28–30°C for 7–14 days in the dark. Conidia were collected in 30% or 15% glycerol and stored at −20°C for routine work or at −80°C for long-term storage.

Protoplast isolation

Fungal protoplasts were prepared using an enzyme mixture consisting of cellulase:lyticase:chitinase (Sigma, St. Louis, Mo.) at a ratio of 40:1:1 in digesting buffer consisting of 1 M sorbitol, 50 mM trisodium citrate, 50 mM EDTA, pH 8.0, in a 50 ml Erlenmeyer flask. The enzyme mixture was prepared and sterilized by filtration using a 0.22 μm pore diameter membrane filter. Fifteen grams of 16- to 18-h-old mycelia were incubated at 30°C in 20 ml digesting buffer with enzymes. The mixture was shaken vigorously on a rotary shaker (Lab-Line, Melrose Park, Ill.) at 200 rpm for 15 min and then at 120 rpm for 75 min. Protoplasts were separated from mycelial debris by filtration through sterile glass wool into a 50 ml sterile disposable conical tube. Fungal protoplasts were collected by centrifugation for 10 min at 2,050 *g* and 4°C. Protoplasts were washed twice with 10 ml washing buffer: 25 mM Tris HCl, pH 7.5, 25 mM $CaCl_2$, 1.2 M sorbitol. The final pellet was resuspended in 5 ml washing buffer, resulting in a concentration of $\geq 10^9$ protoplasts/ml.

Electroporation

Total genomic DNA was extracted from strain ATCC16365 protoplasts and used for electroporation-mediated transformation. Protoplasts were generated as above and resuspended gently in 15 ml 0.7% low-melting agarose (SeaPlaque, FMC BioProducts, Rockland, Me.) in washing buffer at 45°C, and poured into a glass Petri dish. The agarose gel was cut into 0.5 cm^2 pieces. To release the DNA, the embedded protoplasts were soaked overnight in 10 ml liquefied phenol saturated with 100 mM EDTA. The mixture was gently transferred into two 50 ml sterile disposable conical tubes. An equal volume of chloroform was added and tubes were mixed gently on a rocking plate at minimum speed for 15 min. The phases were allowed to separate and the phenol-chloroform phase was discarded by pipetting without centrifugation. The chloroform extraction was repeated three times. Residual phenol-chloroform was removed by forced-air drying for 1 h. The genomic DNA in agarose was purified by adding 75 μl 10 μg/μl DNAse-free RNase A, and incubating at 37°C for 16 h. The agarose was digested with 124 μl Gelase (Epicentre Technologies, Madison, Wis.) and incubated at 45°C for 60 min, following the manufacturer's instructions. Chloroform (1 vol) was added, mixed gently and the mixture was centrifuged at 6,500 *g* for 20 min. A large-bore pipette was used to transfer the upper phase to a Centricon centrifugal filter (YM-100; Millipore, Bedford, Mass.); TE (10 mM Tris, 1 mM EDTA, pH 8) was added to a final volume of 2 ml. The filter device was spun in a Dynac II centrifuge at 800 *g* for 1 h at room temperature. Large genomic DNA adsorbed on the membrane of the Centricon filter was resuspended in 100 μl TE. DNA was quantified by UV (260 nm) spectrophotometry and the size was estimated by field inversion gel electrophoresis gels. The gel conditions were: 0.8% SeaKem LE agarose in 0.5× TBE buffer (45 mM Tris, 45 mM Borate, 1 mM EDTA), 40 s initial time, 40 s final time, 24 h running time using a 2015 Pulsaphore electrophoresis unit (Phamacia LKB, Uppsala, Sweden). Whole lambda DNA and lambda DNA digested with *Bgl*I was used as a DNA size standard.

Samples (400 μl) of strain KB20M1 protoplast suspension ($\geq 10^9$ protoplasts/ml) in protoplast buffer (25 mM Tris HCl, pH 7.5, 25 mM $CaCl_2$, 1.2 M sorbitol) were mixed with 2 μg large DNA fragments from strain ATCC16365 for each treatment. Controls without DNA were included for each set of conditions. Two sets of samples were made, with or without polyethylene glycol (PEG 3640) at a final concentration of 28%, and each set was performed in duplicate. The voltage settings used were 200; 300; 350; 400; 500; 700; 1,000; 1,200; 1,500; 2,000 and 2,500 V.

The protoplast suspension was chilled on ice for 10 min before electroporation. Electroporation cuvettes with a 2 mm gap were used with an Electroporator 2510 (Eppendorf, Westbury, N.Y.). After the electric pulse, the sample was kept on ice for 10 min, then transferred with a sterile glass Pasteur pipette into 10 ml regeneration broth, (complete medium plus 1.2 M sorbitol) in a 50 ml Erlenmeyer flask and incubated at room temperature overnight. Molten regeneration medium (40 ml) plus 2% agar were added, mixed well and the mixture poured into 9-cm-diameter Petri dishes (ca. 15 ml per plate). The cultures were incubated at 28°C in the dark for 16 h.

Because of the electrical resistance of the protoplast suspensions, the actual voltage obtained, as reported by the electroporation

instrument, was less than the voltage settings. In samples without PEG, the average realized voltages were 51% of the voltage setting; with PEG, the average realized voltages were 55% of the setting.

Analysis of color-expressing isolates

Following electroporation, colonies expressing color were identified and isolated as monoconidial isolates by two cycles of serial dilution of conidia in water (1:10–1:10^5) and plating onto potato dextrose agar.

Growth of color variants was compared to strains KB20M1 and ATCC16365. All cultures were grown on 15 ml medium in 9 cm Petri plates. Media used were 25% glycerol nitrate agar, malt extract agar, Czapeks yeast agar, and potato dextrose agar [10]. Plates were incubated for 7 days at 30°C in the dark. Conidial sizes, based on ten randomly selected conidia from each isolate, were compared using an ocular micrometer (600×) and colony sizes were measured using a ruler.

The *Monascus* color variants were qualitatively examined for pigment production on eight solid, and in two liquid, media. The solid media were wheat bread, potato cubes, rice grains, soluble starch agar, and 0.1×, 0.25×, 0.5× or 1× potato dextrose agar. The liquid media were potato dextrose broth and 3% cassava starch plus 4% soy flour.

To look for DNA polymorphisms in the color variants, the random amplified polymorphic DNA (RAPD) [18] technique was applied to DNA from both the recipient and donor strains, and the color variants. Primers used were kits Gen1-80 and Gen2-80 from Sigma-Genosys (St. Louis, Mo.); each kit consists of ten primers of 80% or more G + C. Reaction conditions were: 100 ng template (total genomic) DNA, 0.2 mM dATP, dCTP, dGTP and dTTP; 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20, 3.0 mM $MgCl_2$, 500 nM primer and 1 U Biolase DNA polymerase (Bioline, Springfield, N.J.) in a final volume of 50 μl. PCR conditions were 95°C for 3 min, then 50 cycles of 92°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min. The resulting products were resolved on 1% agarose (SeaKem LE, FMC Bioproducts) in 0.5× TBE.

Optical absorbances of pigments produced by color variants were scanned using a scanning spectrophotometer (Model 530, Jasco, Easton, Md.). Pigments were extracted from the fungus cultured for 21 days on autoclaved rice grains. Ten rice grains from each culture were soaked in 1 ml 80% ethanol in 2 ml microcentrifuge tubes and vortexed at the highest speed of a benchtop vortex mixer for 5 s. Tubes were incubated at room temperature for 20 min, then further vortexed at the highest speed for 5 s every 5 min for an additional 20 min, then centrifuged at 9,500 *g* for 5 min. The supernatants were transferred to fresh tubes and optical densities determined over the wavelength range of 200–600 nm.

Results

Electroporation of albino strain KB20M1 with genomic DNA from red strain ATCC16365

High molecular weight genomic DNA isolated from ATCC16365 was analyzed by field inversion gel electrophoresis, and showed an average length of 15–20 kb (data not shown). Eight isolates that expressed color were obtained following electroporation of albino protoplasts in the presence of this wild-type DNA. All eight colonies were isolated from three electroporation recovery plates on the basis of color expression, 64 days after electroporation (Fig. 1). Six of the color variants

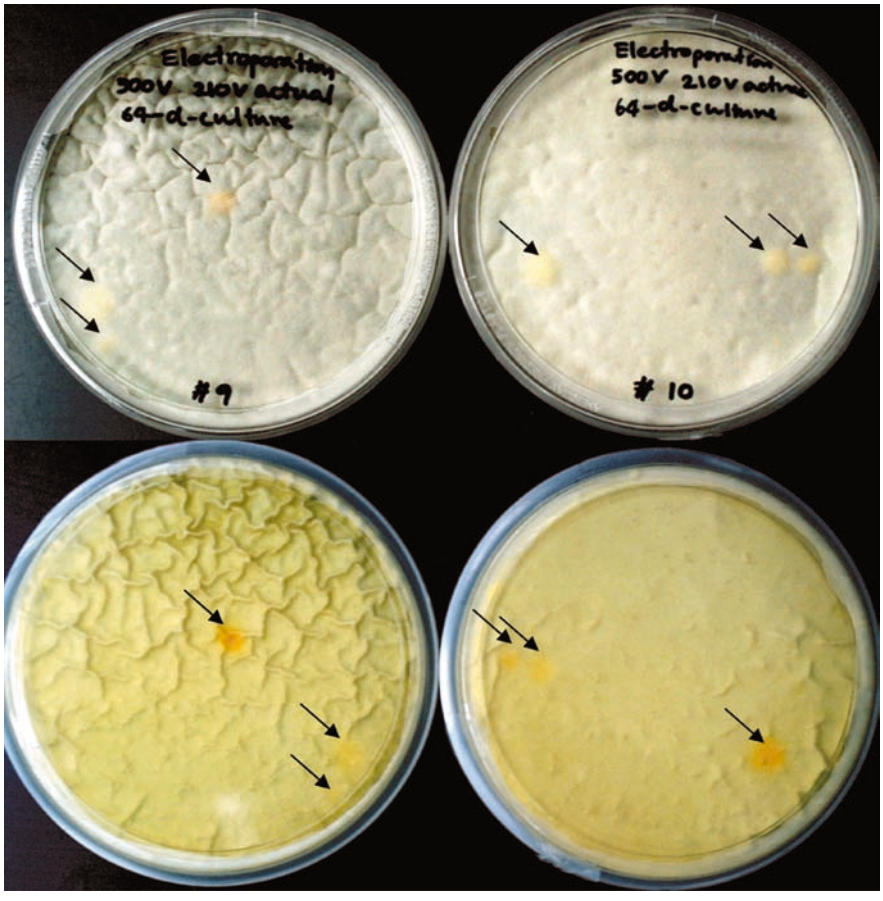

Fig. 1 Colonies of *Monascus purpureus* expressing pigmentation after 64 days growth on medium. The front (top row) and corresponding back (bottom row) of the Petri plates are shown. *Arrows* indicate colored areas

were from two independent trials of electroporation with 500 V without PEG, with a frequency of 1.5 color variants per 1 μg DNA. Two color variants were from the two trials of electroporation with 1,000 V with PEG; frequency = 0.5 color variants/μg DNA. The realized voltages reported by the electroporator were 210 V and 570 V for the 500 V and 1,000 V treatments, respectively. Control plates lacking DNA and all other treatments did not yield color-expressing isolates.

Although the development of color in the variants was slower and less intense than in the type culture, the color variants consistently expressed red color on all media tested except wheat bread and potato. Microscopic examination revealed the pigments to be intracellular, as is normal with *M. purpureus* (Fig. 2). The pigmentation that occurred in liquid media usually occurred in the dry areas on the inner wall of the flask above the shaking broth. These results indicated that expression of the genetic changes that resulted in pigmentation of the color variants was highly affected by environmental factors such as carbon and nitrogen source, aeration, and water content.

All color variants expressed substantial pigmentation when grown on autoclaved rice grains. Spectrophotometric analysis of ethanol extracts from rice grain cultures of the color variants showed some variation in absorbance spectra from 300 nm to 500 nm (Fig. 3). All color variants had absorbance spectra different from the albino. At least two absorbance peaks were seen, at 390 nm and 410–430 nm, respectively (Fig. 3). These peaks were not found in extracts from the albino strain. The absorbance of the extracts from EP10-2 and EP20-2 at 410–430 nm appeared to differ from the other isolates (Fig. 3), suggesting that novel pigments may have been produced by those isolates.

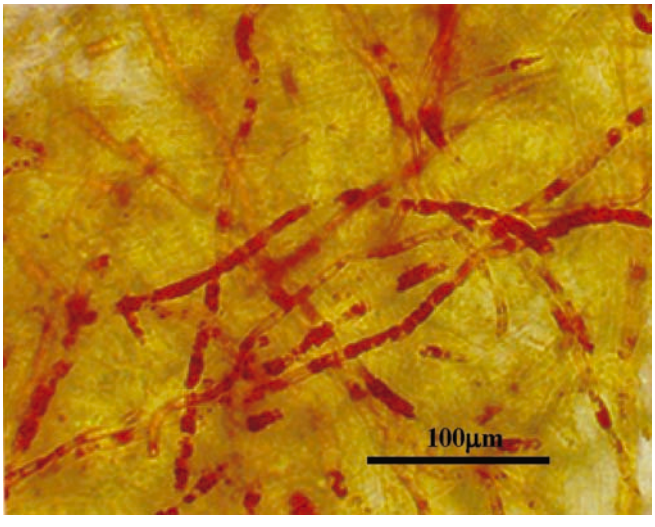

Fig. 2 Pigment expression within the mycelia of isolate EP9-2, derived from electroporation of protoplasts in the presence of genomic DNA, grown in potato dextrose broth for 10 days

DNA analysis of color-expressing variants

To identify DNA polymorphisms in the eight electroporation-derived color variants, RAPD analyses were performed with 80% GC primers. Variation was seen in the RAPD products (Fig. 4), but when these novel fragments were used as probes in Southern blot hybridization, no extra hybridizing bands were found (data not shown). Slight size differences among hybridizing restriction fragments were seen in the eight color variants when probed with three different RAPD fragments. These size polymorphisms are suggestive of homologous DNA replacement, which may indicate that the albino protoplasts had undergone a homologous replacement transformation event in each isolate. Homologous replacement is expected from transformation with large regions of highly homologous DNA [4].

The growth of the eight color variants and the recipient and donor strains were compared on four media. Colony growth after 7 days on malt extract agar of all eight of the color variants was significantly greater than that of the recipient and donor strains (Table 1). On potato dextrose agar and Czapeks yeast agar, some of the color variants grew larger colonies than the recipient and donor strains, but none of the colony diameters were significantly different from the recipient strain on 25% glycerol nitrate agar (Table 1).

Discussion

Electroporation of protoplasts from albino *M. purpureus* strain KB20M1 in the presence of genomic DNA from a

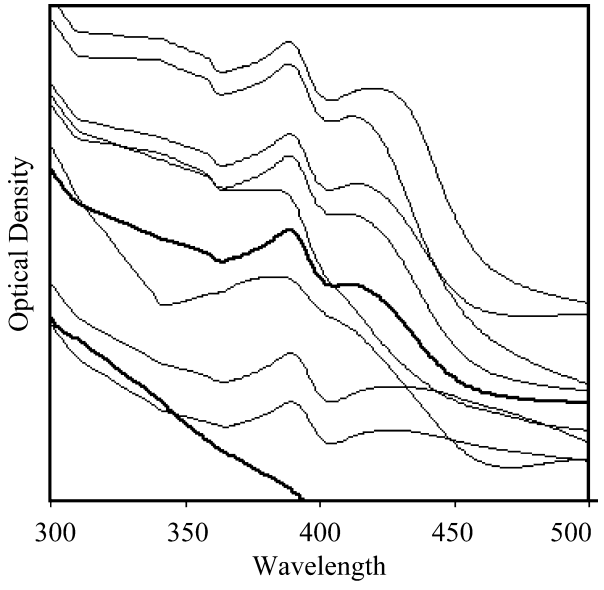

Fig. 3 Optical density traces of pigments extracted from isolates of *M. purpureus* derived from electroporation of albino strain KB20M1 in the presence of genomic DNA of the red type-culture. The order of isolates, from top to bottom at the major peak at 390 nm is: EP9-3, EP9-1, EP10-3, EP10-1, EP20-1, ATCC16365 (*bold*), EP9-2, EP10-2, EP20-2, KB20M1 (*bold*)

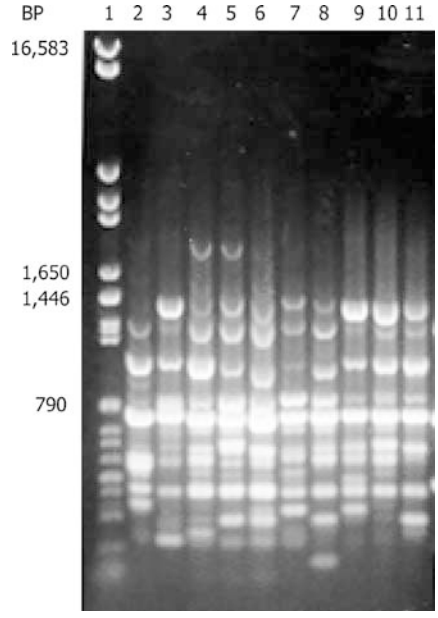

Fig. 4 Polymorphisms among random amplified polymorphic DNA (RAPD) products of eight isolates derived from electroporation of albino strain KB20M1 in the presence of genomic DNA of the red type culture using primer GEN1-80-01 (GCACCCGACG). Lanes: *1* Lambda phage *Bgl*I marker fragments, *2–11* products from strains ATCC16365, KB20M1, EP9-1, EP9-2, EP9-3, EP10-1, EP10-2, EP10-3, EP20-1, and EP20-2, respectively

Table 1 Average diameter of color-expressing *Monascus purpureus* colonies found after electroporation of protoplasts from albino strain KS20M1 in the presence of genomic DNA from red strain ATCC16365. *CYA* Czapeks yeast agar, *G25N* 25% glycerol nitrate agar, *MEA* malt extract agar, *PDA* potato dextrose agar

Colony diameter[a] on culture medium				
Isolate[b]	CYA	G25N	MEA	PDA
ATCC16365	34.0 ± 0.90	20.9 ± 1.4	30.8 ± 4.5	28.0 ± 0.2
KB20M1	36.2 ± 1.6	14.2 ± 1.3	35.8 ± 1.1	33.9 ± 0.6
EP9-1	38.5 ± 1.2	13.2 ± 0.5	51.6 ± 1.9	41.3 ± 0.4
EP9-2	36.5 ± 0.9	15.1 ± 0.4	50.4 ± 0.9	42.0 ± 0.7
EPP-3	42.6 ± 0.8	13.1 ± 0.4	47.4 ± 4.1	41.2 ± 0.5
EP10-1	42.1 ± 0.9	15.2 ± 0.1	54.4 ± 0.9	41.9 ± 0.6
EP10-2	40.2 ± 0.8	14.8 ± 0.4	50.8 ± 3.6	31.9 ± 0.8
EP10-3	38.5 ± 2.6	13.8 ± 1.1	52.8 ± 0.6	36.2 ± 0.4
EP20-1	37.3 ± 1.2	14.0 ± 0.5	52.8 ± 2.2	37.9 ± 0.6
EP20-2	38.4 ± 1.0	14.4 ± 1.2	53.4 ± 1.0	34.2 ± 0.3
LSD[c]	2.36	1.61	4.45	1.91

[a]Average diameter (mm), followed by the standard error ($n = 3$) of fungal colonies after 7 days growth on the specified medium at 28°C in the dark
[b]Isolates with an EP prefix were color variants derived from electroporation of strain KB20M1 in the presence of genomic DNA isolated from strain ATCC16365
[c]Least significant difference value with $P = 0.05$

red donor strain resulted in isolates that expressed color. This result probably originated from the expression of genetic material from the red strain in the albino mutant (genetic transformation), reversion of the albino mutation(s), or the activation of alternative pathway(s), e.g., via new mutations, leading to visible color production. The color variants were produced only under two of the electroporation conditions tested, and only in trials including the wild-type DNA. The albino strain KB20M1 has not been seen to revert to color production in more than 10 years of study (Busaba Yongsmith, personal communication). Hence, it is likely that the color variants arose as a result of introduced DNA.

Color expression was not observed until 64 days after electroporation, at which time the colonies expressing color were about 6 mm (or less) in diameter (Fig. 1). Therefore, the expression of color did not begin until several weeks after electroporation, suggesting an extended period of time was necessary for stabilization and activation of the genetic change(s) responsible for pigment production. The pigment production capability of these isolates has been stable through numerous cycles of subculturing, suggesting stabilized integration of introduced DNA, or a stable mutation.

Regardless of the physical origin of these altered phenotypes, their occurrence indicates that gene(s) are expressed in the color variants that are not expressed in the albino mutant. The color variants produced in this study are a step towards the goal of identifying genes whose expression is necessary for the function of one or more pathways vital to pigment production in this industrial food fungus.

Acknowledgements The authors thank Busaba Yongsmith for providing strain KB20M1 and for technical advice, The Royal Golden Jubilee Ph.D. Program of the Thailand Research Fund, and Kasetsart University Graduate School for partial financial support.

References

1. Berset C, Clermont HS, Cheval S (1995) Natural red colorant effectiveness as influenced by absorptive supports. J Food Sci 60:858–861
2. Bowyer P, Osbourn AE, Daniels MJ (1994) An "instant gene bank" method for heterologous gene cloning: complementation of two *Aspergillus nidulans* mutants with *Gaeumannomyces graminis* DNA. Mol Gen Genet 242:448–454
3. Butler PD, Moxon ER (1990) A physical map of the genome of *Haemophilus influenzae* type b. J Gen Microbiol 136:2333–2342
4. Chaveroche MK, Ghigo JM, d'Enfert C (2000) A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. Nucleic Acids Res 28:E97
5. Chu SW, Poon YK (1993) Submerged production of *Monascus* pigments. Mycologia 85:214–218
6. Cline SW, Schalkwyk LC, Doolittle WF (1989) Transformation of the archaebacterium *Halobacterium volcanii* with genomic DNA. J Bacteriol 171:4987–4991
7. Fink-Gremmels J, Leistner L (1989) Biological effects of *Monascus purpureus*. Fleischwirtschaft 69:115–122
8. Fink-Gremmels J, Dresel J, Leistner L (1991) Use of *Monascus* extracts as an alternative to nitrite in meat products. Fleischwirtschaft 71:1184–1186
9. Gems D, Aleksenko A, Belenky L, Robertson S, Ramsden M, Vinetski Y, Clutterbuck AJ (1994) An 'instant gene bank' method for gene cloning by mutant complementation. Mol Gen Genet 242:467–471
10. Hawksworth DL, Pitt JI (1983) A new taxonomy for *Monascus* species based on cultural and microscopical characters. Aust J Bot 31:51–61

11. Ho BW, Guo JJ, Fang YC (1973) Studies on the screening of glucoamylase producing strains of *Monascus* and their fermentation conditions. Acta Microbiol Sinica 13:142–150
12. Iizuka H, Lin CR (1981) On the genus *Monascus* of Asia and its specific characteristics. Adv Biochem 2:555–561
13. Kelly R, Register E, Sosa M (1994) Heterologous transformation of *Zalerion arboricola*. Curr Genet 26:217–224
14. Lorito M, Hayes CK, Di Pietro A, Harman GE (1993) Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. Curr Genet 24:349–356
15. Ma J, Li Y, Ye Q, Li J, Hua Y, Ju D, Zhang D, Cooper R, Chang M (2000) Constituents of red yeast rice, a traditional Chinese food and medicine. J Agric Food Chem 48:5220–5225
16. Teng SS, Feldheim W (2000) The fermentation of rice for anka pigment production. J Ind Microbiol Biotechnol 25:141–146
17. Teng SS, Feldheim W (2001) Anka and anka pigment production. J Ind Microbiol Biotechnol 26:280–282
18. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535
19. Wong HC, Bau YS (1977) Pigmentation and anti-bacterial activity of fast-neutron and X-ray-induced strains of *Monascus purpureus* Went. Plant Physiol 60:578–581
20. Wong HC, Lin C, Koehler PE (1981) Regulation of growth and pigmentation of *Monascus purpureus* by carbon and nitrogen concentrations. Mycologia 73:649–654
21. Wong HC, Koehler PE (1981) Production and isolation of an antibiotic from *Monascus purpureus* and its relationship to pigment production, control of microbial contamination, and food colorant. J Food Sci 46:589–592
22. Wong HC, Koehler PE (1983) Production of red water-soluble *Monascus* pigments. J Food Sci 48:1200–1203
23. Yongsmith B, Krairak S, Bavavoda R (1994) Production of yellow pigment in submerged culture of a mutant of *Monascus* spp. J Ferment Bioeng 78:223–228
24. Yongsmith B, Kitprechavanich V, Chitradon L, Chaisrisook C, Budda N (2000) Color mutants from *Monascus* sp. KB9 and their comparative glucoamylases on rice solid culture. J Mol Catal B 10:263–272